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Plasma hydrogenated cationic detonation nanodiamonds efficiently deliver to human cells in culture functional siRNA targeting the Ewing sarcoma junction oncogene

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ABSTRACT

The expression of a defective gene can lead to major cell dysfunctions among which cell proliferation and tumor formation. One promising therapeutic strategy consists in silencing the defective gene using small interfering RNA (siRNA). In previous publications we showed that diamond nanocrystals (ND) of primary size 35 nm, rendered cationic by polyethyleneimine-coating, can efficiently deliver siRNA into cell, which further block the expression of *EWS/FLI-1* oncogene in a Ewing sarcoma disease model. However, a therapeutic application of such nanodiamonds requires their elimination by the organism, particularly in urine, which is impossible for 35 nm particles. Here, we report that hydrogenated cationic nanodiamonds of primary size 7 nm (ND-H) have also a high affinity for siRNA and are capable of delivering them in cells. With siRNA/ND-H complexes, we measured a high inhibition efficacy of *EWS/FLI-1* gene expression in Ewing sarcoma cell line. Electron microscopy investigations showed ND-H in endocytosis compartments, and especially in macropinosomes from which they can escape before siRNA degradation occurred. In addition, the association of *EWS/FLI-1* silencing by the siRNA/ND-H complex with a vincristine treatment yielded a potentiation of the toxic effect of this chemotherapeutic drug. Therefore ND-H appears as a promising delivery agent in anti-tumoral gene therapy.

1. Introduction

In recent years, nanodiamonds (NDs) have gained increasing attention as powerful nanoplatform with fluorescent labeling capacities for both diagnostic and therapeutic purposes [1,2]. Indeed, NDs have properties that make them highly suitable for biomedical applications: several *in vitro* [3] and *in vivo* [4] studies have shown that NDs are non-cytotoxic with an enhanced cell tolerance compared to other carbon nanoparticles, and their carbon-related surface chemistry is interesting for covalent or non-covalent functionalization of therapeutic, targeting or labeling moieties (drugs, oligonucleotides, proteins, fluorescent dyes) [5,6]. In this context, small interfering RNA (siRNA) is a powerful agent for gene inhibition, but its activity is limited by a poor capacity to penetrate into cells and by the degradation by nucleases. For this reason, several groups have developed vectors to deliver efficiently oligonucleotides [7] and siRNA to cells [8]. In previous studies we showed that synthetic NDs of primary core size around 35 nm, produced by high-pressure and high temperature synthesis (HPHT), can deliver active siRNA to cells in culture resulting in inhibiting the expression of Ewing sarcoma junction oncogene *EWS/FLI-1* in Ewing human cells [9,10]. These NDs were carboxylated by strong acid cleaning resulting in a negative surface charge at pH = 7. They were then coated with cationic polymers (polyethylene-imine [PEI] or polyallylamine hydrochloride [PAH]) so that negatively charged siRNA can bind to them by electrostatic interaction. This strategy has revealed to be efficient with a low

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toxicity for the transfected cell and a good gene inhibition. However, it cannot be translated to animals because of the vector size (further increased to about 120–130 nm after polymer coating [9]) preventing its elimination in urine after intravenous injection [11]. Moreover, since the polymer is non-covalently bound to the ND vector, it may dissociate from its surface after injection to the animals, taking the siRNA away with it, which results in a loss of the expected gene inhibition.

In this study, we used intrinsically cationic nanodiamonds [12] of 7 nm primary core size, which could be compatible with *in vivo* applications. Indeed, one can expect their elimination by kidney [11]. These nanodiamonds produced by detonation of high energy explosives (hexogen/RDX) [13] were exposed to a micro-wave hydrogen plasma leading to an efficient cleaning of oxygen groups, a removal of non-diamond carbon and the formation of C sp^3-H terminations as previously reported [12,14,15]. After plasma exposure, hydrogenated detonation ND (ND-H) were dispersed in water forming a stable suspension with a positive zeta potential of +50 mV favorable to nucleic acids electrostatic binding. The origin of the positive Zeta potential was recently explained by a surface transfer doping mechanism [16]. In this work, we show that siRNA binds to ND-H, and we demonstrate that the siRNA/ND-H complex can efficiently deliver siRNA promoting *EWS/FLI-1* targeted gene silencing into human Ewing sarcoma cells in culture. These results establish a first step towards applications to small animals of ND vector that could be eliminated in urine owing to its ultrasmall size.

2. Experimental section

2.1. Cationic ND-H and anionic ND-COOH preparation and characterization

Detonation nanodiamonds were provided by the NanoCarbon Institute Co., Ltd. (Japan). Hydrogenation of nanodiamonds was achieved by microwave-enhanced plasma treatments. A mass of 80–100 mg of NDs was deposited in a quartz tube and inserted in a plasma Downstream source (Sairem SAS, France). High purity hydrogen gas (>N70) was injected in the tube at a pressure of 15 mbar and plasma was generated in the quartz tube at a micro-wave power of 300 W (2.45 GHz). During the plasma, the tube was air-cooled. NDs were exposed to hydrogen plasma for 15 min and were cooled down under hydrogen, leading to ND-H. More details were previously reported [12]. ND-H were then dispersed in ultrapure water by sonication (UP400S, 300 W, 24 kHz, Hielscher Ultrasonics GmbH, Germany) for 2 h under cooling. Larger aggregates were removed from the solution by centrifugation at 4000 rpm (30 min or 2 h depending on the sample). Oxidized NDs (ND-COOH) were obtained by air annealing (400 °C for 2 h) and dispersed in ultrapure water by the same sonication procedure than ND-H. Size and Zeta potential measurements of ND-H and ND-COOH suspensions were performed in ultrapure water on a Nanosizer ZS (Malvern, UK) in the back scattering configuration (173°).

2.2. siRNA binding to ND-H

The binding capacity of cationic hydrogenated ND-H was studied by mixing 28 ng of siRNA in 30 μL of 10 mM HEPES buffer at pH = 7.2 and 100 mM NaCl with increasing concentration of ND-H ranging from 0 to 0.1 μg/μL. After 10 min incubation at room temperature, the solution was centrifuged at speed 13,000 rpm, for 10 min and at 15 °C. Then 30 μL of supernatant was mixed with 30 μL of 1 μg/mL ethidium bromide (Sigma–Aldrich, USA) and fluorescence was recorded with a fluorescence plate reader with excitation wavelength of 525 nm, and detection range 580–640 nm (GloMax®-Multi, Promega). Experiments were performed in triplicate. siRNA labeled with FITC dye (siRNA-FITC) was used to identify the location of siRNA in cell by epifluorescence microscopy.

2.3. Cytotoxicity

A673 human Ewing sarcoma cell line was a generous gift from Dr. Elizabeth R. Lawlor (University of Michigan, USA). Cells were grown in DMEM medium supplemented by 10% of fetal bovine serum (Gibco, USA) and 1% penicillin-streptomycin antibiotics (Gibco). One day before treatment, 2×10^3 cells by well were plated in 96 wells and incubated at 37 °C, 5% CO_2 in moistly atmosphere. The medium was then removed and replaced by 100 μL medium containing increasing concentrations of either ND-H or ND-COOH from 0 to 0.3 mg/mL. Two series were treated with ND mixed with 50 nM siRNA. Cells were incubated for 48 h and the cell viability was then determined using a MTT assay. Briefly, 10 μL of 5 mg/mL MTT in PBS buffer were added and cells were incubated for 2 h. Then, 100 μL of lysis buffer (10 mH HCl, 10% SDS) was added overnight and the produced formazan was quantified on cell suspension by absorbance measurement at 570 nm wavelength using a micro plate reader (ELx808, BioTek, USA). Experiments were performed in 8 replicates. Data were corrected from the absorbance/scattering of free ND suspension. For such correction, a 96 wells plate is seeded with just ND solution at increasing concentrations and MTT treatment is performed as for cell cytotoxicity test. Then optical density is measured with the microplate reader and the value corresponding to each ND concentration is subtracted to the one measured for cells treated with the same ND concentration. Results are expressed as % of untreated cells.

2.4. siRNA delivery to cells: epifluorescence microscopy observations

A673 cells (2×10^5 cells per well) were seeded 24 h before the addition of cationic ND in 12 wells plate with 18 mm diameter coverslips at the bottom, and containing 500 μl DMEM medium (Gibco) with 10% bovine calf serum (Gibco) and 1% Penicillin/streptomycin solution (Gibco). The culture was done at 37 °C, 5% CO_2 in moistly atmosphere. The medium was then discarded and replaced by 500 μL of the same medium or serum free Opti-MEM medium (Gibco) containing ND-H/siRNA-FITC fluorescent conjugate at mass ratio of 10, 25, 50 and 75 (ND/siRNA, μg/μg), at final siRNA concentration of 50 nM. The mass-ratio corresponds to the mixed masses of NDs and siRNA. The ND-H/siRNA-FITC were incubated with cells for 4 h at 37 °C, 5% CO_2 in moistly atmosphere. Then, the cells were washed twice with 1 mL PBS and fixed with 4% formaldehyde in PBS for 20 min at room temperature. The cells were washed with PBS and mounted on microscope slide with Dapi Fluoromount G (SouthernBiotech, USA). The internalization of ND-H/siRNA-FITC by the cells was monitored with an epifluorescence microscope (Observer, Zeiss, Germany).

2.5. siRNA delivery to cells: confocal microscopy and fluorescence spectroscopy

Confocal raster scans (ESI Fig. S2) were done with a home-made microscope described in Ref. [17], equipped with single photon counting module capable of detecting single nitrogen color center. Neutraly charged nitrogen-vacancy color center emission having a maximum around 615 nm wavelength, was detected in the microscope red channel. The confocal microscope excitation source is a continuous wave (cw) solid-state laser (561L-100-COL-PP, Oxxius SA, France) emitting excitation at the wavelength 561 nm. The red detection channel consists in a longpass detection/emission filter with a steep edge at 580 nm and an optical density larger than 6 at 561 nm (BLP01-561R-25, Semrock, USA). The microscope green detection channel allowing to detect FITC dye fluorescence is composed of a cw solid-state laser emitting at 488 nm wavelength (Sapphhire 488-50, Coherent Inc., USA), and a detection/emission bandpass filter centered on 525 nm (ET525/50, Chroma Corp., USA). Both 561 nm and 488 nm beam were focused onto the sample via an immersion oil ×60, numerical aperture 1.40 microscope objective (CFI Plan Apochromat DM60x oil, Nikon, Japan) after their combination and reflection onto a double-band dichroic filter (Di01-R488/561, Semrock). The fluorescence spectrum displayed on Fig. S2f was acquired with an imaging spectrograph (MicroHR MHRA-1X-AMS equipped with Symphony CCD-1024x256-BIUV-STE Open electrode cooled CCD array detector, Horiba Jobin Yvon, France).

2.6. siRNA delivery to cells: TEM studies

For TEM studies, the cells were seeded in 12 wells plates containing a coverslip at 8×10^4 cells per wells 24 h before the addition of ND. Then the medium was discarded and replaced by 500 μL of DMEM medium containing 10% bovine calf serum or free serum OptiMEM medium containing 40 μg/mL of NDs-H. The cells were incubated for 3 h at 37 °C, 5% CO_2 in a moistly atmosphere. The medium was discarded and replaced by 1 mL of 2% glutaraldehyde (EMS, Hatfield, PA, USA) in 0.1 M cacodylate buffer pH = 7.4, for 1 h at room temperature. Cells were post-fixed for 1 h at room temperature with 1% osmium tetroxide and 1.5% potassium ferrocyanide (Sigma–Aldrich, USA) in cacodylate buffer (EMS, Hatfield, PA, USA). They were dehydrated by increasing concentrations of ethanol and finally embedded in Epon812 epoxy resin (EMS, Hatfield, PA, USA). The polymerization was carried out by heating the sample during 72 h at 56 °C. Samples were then sectioned with a microtome (thickness 90 nm), and the sections were collected on collodion-carbon-coated copper grids. Sections were contrasted using aqueous uranyl acetate 2% (w/v) (Merck, USA) and lead citrate solutions (Reynold's stain). The samples were observed with Zeiss 902 TEM in the filtered zero loss modes using a CCD array detector (Megaview III, Olympus, Japan) coupled to the SIS software (Olympus).

2.7. Inhibition of EWS/FLI-1 gene expression

24 h before treatment, 5×10^4 A673 cells are seeded per wells in 12 wells plate with 500 μL DMEM medium (Gibco) containing 10% bovine calf serum and 1% penicillin/streptomycin (Gibco) and incubated at 37 °C, 5% CO_2 in moistly atmosphere. Then, medium was removed and replaced by 450 μL of same medium and 50 μL of 10 mM Hepes pH 7.2, 100 mM NaCl containing free siRNA or NDs-H bound siRNA targeted toward EWS/FLI-1 or a control sequence at 50 nM final concentration. siRNA/ND mass ratio was 50 (w/w). Cells were incubated for 24 h and total RNA was extracted by Trizol (Invitrogen, USA) methods. Briefly, the cell culture medium is discarded first. Cells are then washed with PBS and lysed with 800 μL of Trizol solution. Finally, the cells are scrapped and 160 μL of chloroform/isoamyl alcohol